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13. ABSTRACT (Maximum 200 Words)
Rd51 is important for repairing double-strand breaks in DNA by recombinational repair, a pathway that utilizes a homologous template usually provided by the sister chromatid for somatic cells. This function may be essential since mammalian cells deleted for Rad51 exhibit chromosomal instability, are unable to sustain proliferation and senesce or die. In order to function, Rad51 associates with Brca2, a protein important for suppression of breast cancer. Rad51 associates with Brca2 in two domains: the most amino- terminal domain is mediated by the BRC motifs (encoded in exon 11) and in the most COOH- terminal domain by a single region (encoded by exon 27). COOH terminal deletions that remove some but no all of these regions increase replicative senescence and sensitivity to ionizing radiation and cross-linking agents, suggesting that the Rad51 – Brca2 association is biologically important. Here we describe a peptide, derived for the Rad51 interacting region encoded in exon 27 that biochemically interacts with a Rad51 filament that is associated with DNA. Additionally, exposure to the peptide causes a reduction of replication, disruption of Rad51 foci and programmed cell death. We propose that the peptide forms a nonproductive association with Rad51 that inhibits Rad51 function. Such a peptide may serve useful as an anti-cancer agent.

14. SUBJECT TERMS
DNA double-strand break; recombinational repair; ionizing radiation RAD51; BRCA2

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Introduction

BRCA2 is important for suppressing breast cancer. Women with one mutant copy of BRCA2 are predisposed to breast cancer with loss of heterozygosity being an important step in the oncogenic process. BRCA2 functions to repair DNA double-strand breaks by recombination. At the cellular level, disruption of recombination impedes proliferation and induces either senescence or apoptosis. In addition, BRCA2-deficient cells are sensitized to agents that generate DNA double-strand breaks like ionizing radiation and DNA crosslinking agents. Thus, disruption of the association of BRCA2 with RAD51 should be efficacious in the treatment of cancer by acting as an adjuvant to radiation therapy and chemotherapy by decreasing the cell's ability to repair damage caused by these DNA damaging agents. Based on this reasoning we have developed a small peptide derived from a region encoded in exon 27 of BRCA2 that interacts with RAD51. Exposure to this peptide decreases proliferation and initiates apoptosis for cancer-derived cells grown in tissue culture. We propose to characterize this peptide by determining its cytotoxic effect on a variety of cells derived from tumors that are both radiosensitive and radioresistant. This peptide could prove to be an effective adjuvant to ionizing radiation and DNA crosslinking agents that would be especially useful for treating tumors that are resistant to these therapies and for lowering doses of these cytotoxic drugs, thereby reducing their deleterious side effects.

Body

RAD51 is important for repairing double-strand breaks in DNA by recombination (12); interestingly, this function is likely to be essential since mammalian cells deleted for RAD51 exhibit chromosomal instability, are unable to sustain proliferation and senesce or die (4). In order to function, RAD51 associates with BRCA2 (11), a protein important for suppression of breast cancer (13). RAD51 associates with BRCA2 in two domains: the most amino - terminal domain is mediated by the BRC motifs encoded in exon 11 (1) and in the most carboxy - terminal domain is mediated by a single region encoded in exon 27 (11). COOH terminal deletions that remove some but not all of these regions increase replicative senescence and sensitivity to ionizing radiation and cross-linking agents, suggesting that the RAD51 - BRCA2 association is biologically important (2, 7, 9). We describe in the grant a 26 amino acid peptide (PLPSPVSPICTFVSPAAQKAFQPPRS), derived from the RAD51 interacting region encoded in exon 27 of BRCA2 that biochemically interacts with a RAD51 filament formed on single stranded DNA. Biological activity of this 26mer was assessed by conjugating it to 16 amino acids from the third helix of the Antennapedia homeodomain (antp) that enables proteins to transverse biological membranes. Exposure to antp-26mer causes a reduction of cellular proliferation, disruption of RAD51 foci and programmed cell death when applied to tissue culture cells that were derived from cancer. We propose that antp-26mer forms a nonproductive association with RAD51 that inhibits its function and disables recombinational repair, leading to reduced cellular proliferation and induces either cellular senescence or apoptosis. In addition, compromised RAD51 function would increase sensitivity to ionizing radiation and to other clastogenic agents that are common cytotoxic drugs used to fight cancer. Our goal is to develop this RAD51-interacting peptide as an anti-cancer therapeutic.

During the first year we isolated the smallest active peptide that is important for biological activity, we found that five amino acids (PICTF) are sufficient. These five amino acids when conjugated to antp induce cell death and disrupt rad51 foci. We then performed a substitution

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analysis of antp-PICTF. Importantly, The C-M switch ablates activity (antp-PIMTF), the T-A switch reduces activity (antp-PICAF) while the T-S switch does not alter activity (antp-PICSF).

These peptide derivatives could prove to be beneficial as anti-cancer agents by hindering cellular proliferation and by sensitizing cells to chemotherapeutics and ionizing radiation. In order to determine the effectiveness of these peptides as anti-tumor agents, they were tested for their ability to enhance the treatment of currently used chemotherapeutics and radiation. Towards this goal we first developed a genotoxic screen with mouse embryonic stem (ES) cells, HeLa cells and mouse embryonic fibroblasts (MEF) (Fig. 1) in order to test for possible synergy of the peptide with these agents. The genotoxic screen is a rapid method that determines the cellular dose-response to a wide range of agents that either damage DNA or alter basic cellular pathways important for maintaining genomic integrity. Thus, the integrity of a wide range of DNA repair, chromatin metabolism, and response pathways is tested.

ES cells and HeLa cells were exposed to a low dose of antp-PICTF (2 μM), a low dose of genotoxin and to low doses of both. After five days, cells were counted and a survival fraction determined based on cells exposed to only vehicle. Of the 20 genotoxins, there was significant synergy with a synthetic non-toxic seleno-organic drug called ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) (Fig. 2). Ebselen is a non-toxic seleno-organic drug with antiinflammatory, antiatherosclerotic and cytoprotective properties (10) and is an effective scavenger of organic hydroperoxides, in particular, of lipid hydroperoxides (8). We found that 2 μM antp-PICTF and 15 μM ebselen killed all cells while either agent alone resulted in only a slight reduction in cell number for both ES cells and HeLa cells. We then determined that high Pico molar concentrations of antp-PICTF could significantly reduce cell number in the presence of 15 μM ebselen (Fig. 3). Ebselen had no impact on antp-PIMTF when exposed to HeLa cells (not shown).

The sensitivity of genetically altered cells to antp-PICTF and antp-PIMTF was tested (Fig. 4). HeLa cells were sensitive to only antp-PICTF at a range of 5 μM to 30 μM but were not sensitive to antp-PIMTF even at 100 μM. We found that cells deleted for nonhomologous end joining (NHEJ) or compromised for homologous recombination were hypersensitive to antp-PICTF, but not antp-PIMTF. Cells deleted for NHEJ are null for Ku80 (5, 14) which is essential for NHEJ. Cells deficient for homologous recombination are deleted for Brca2 exon 27 (3, 7). Homologous recombination is essential for cell viability (4); therefore, cells null for homologous recombination can not be maintained in tissue culture and can not be tested. Cells deficient for the RecQ helicase, Bloom's syndrome (Blm)(6), exhibit the same sensitivity to antp-PICTF as control cells. These observations imply that antp-PICTF causes or impairs the repair of DNA double strand breaks since NHEJ and recombination are the two most prominent pathways that repair DNA double-strand breaks. In addition, these genetically altered cells exhibit hypersensitivity to clastogenic agents including cisplatin and γ-radiation.

Currently, we are analyzing the structural conformation of the peptide. We are doing this out of necessity, not design. Over the last year we have had a difficult time obtaining antp-PICTF that is active. We have obtained one batch from Tufts that is not toxic at any dose even with ebselen. We have obtained two batches from Sigma Genesis that has partial activity (kills cells only with ebselen and at a high dose). We have two batches from our own facility at the Health Science Center (HSC) that has normal activity; however, it takes months to get 5 mgs (the first batch took 9 months). We are trying to determine the reason for this difference in activity. We now know that the HSC peptide and the Tufts peptide have slightly different peaks by HPLC. The SG peptide has both peaks (the minor peak is the same as the HSC and the major peak is the same

as the Tufts). We are now testing for racemization of the peptides. It is critical that we understand the difference between these peptides so that we may have a reliable source.

After this is completed we will test biological activity on cells derived from breast cancer and cells that posses either wild type or diminished BRCA2 function. We will also test cells with a variety of genetic backgrounds that may induce cancer. Antp-26mer and its derivatives could be highly efficacious against radio-resistant forms of cancer. These peptides will finally be tested for potential anti-cancer activity in a mouse model prone to mammary carcinoma. In addition, peptides will be tested as possible adjuvants to radiation therapy.
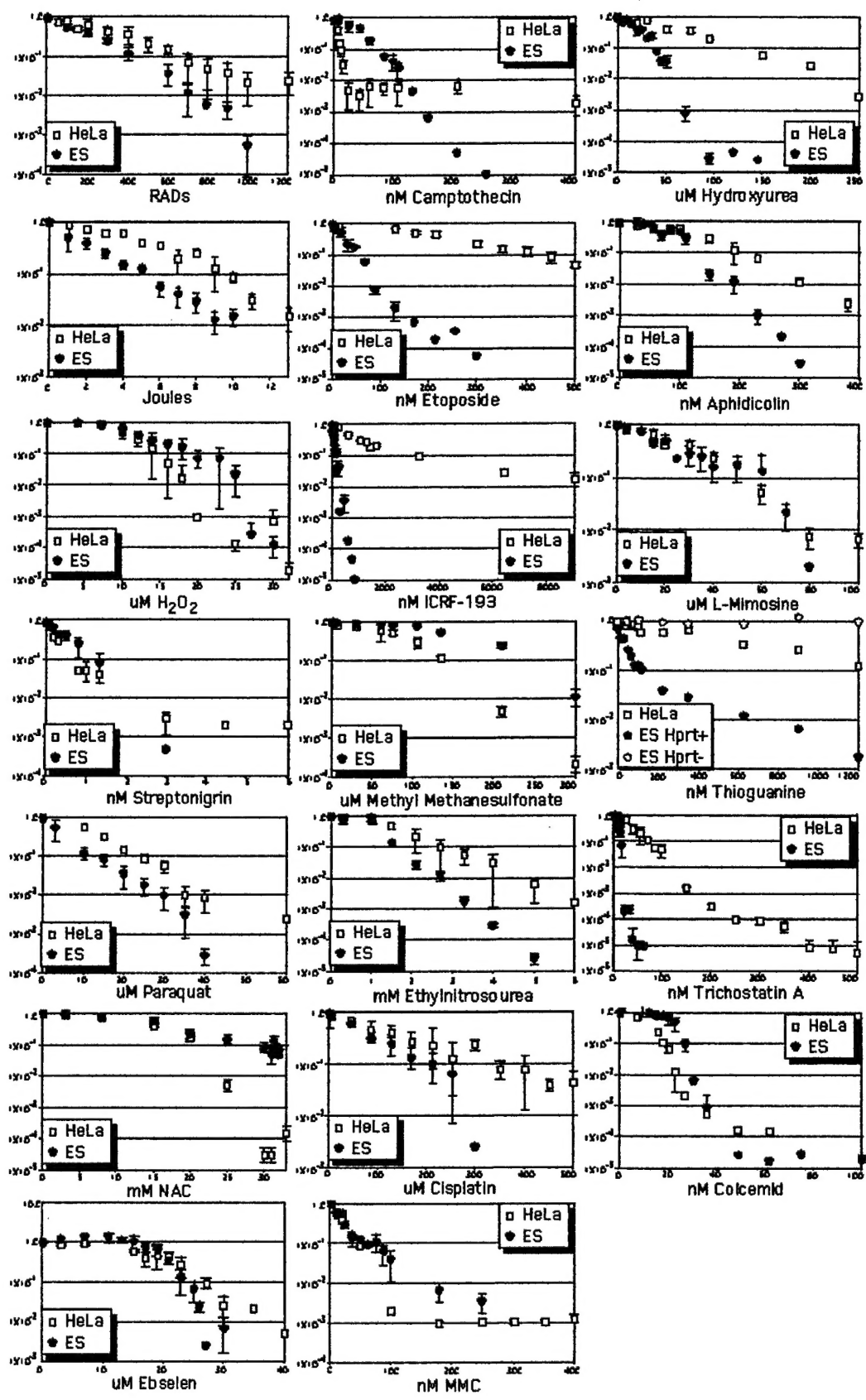
Figure 1. Dose response curves of ES and HeLa cells for the 20 agents that make up the genotoxic screen.
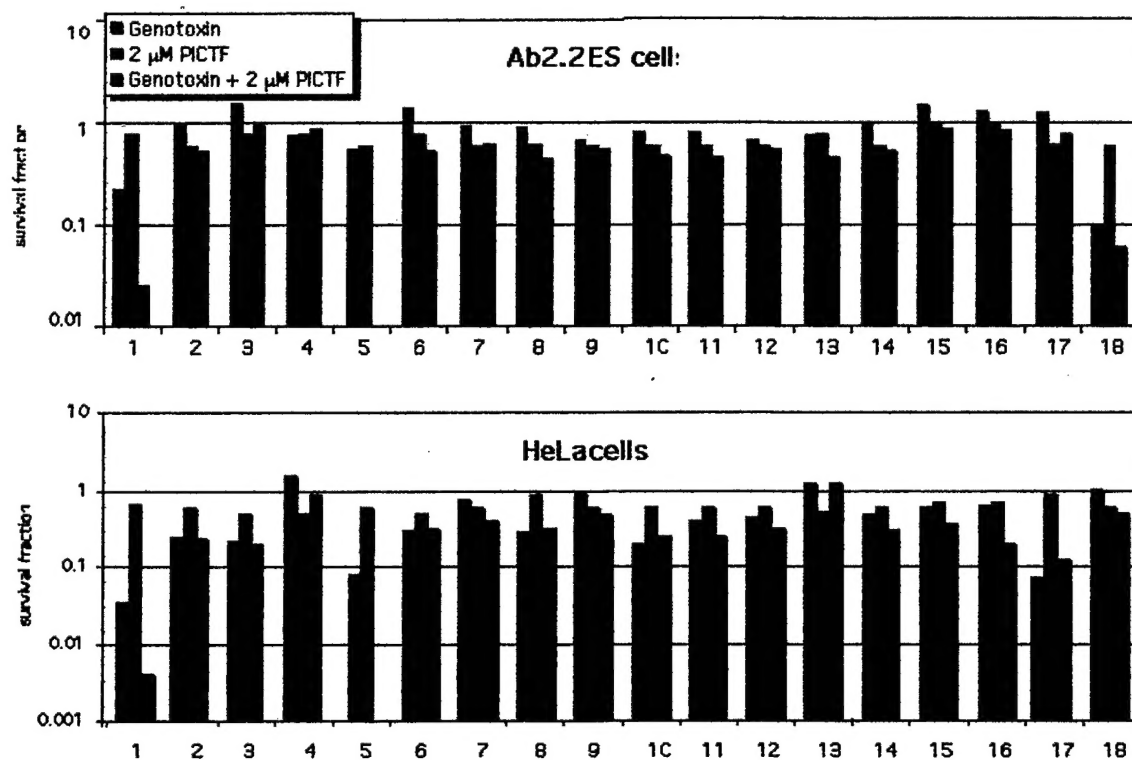
Figure 2. Test for synergy between antp-PICTF and 18 of the 20 genotoxins. There appears to be mild synergy with H_2O_2 and dramatic synergy with ebselen for both ES cells (AB2.2, top) and HeLa cells (bottom). 1. H_2O_2, 2. Streptonigrin, 3. Paraquate, 4. N-acetyl-L-cysteine, 5. Ebselen, 6. Methyl methane sulfonate, 7. Ethylnitrosourea, 8. Mitomycin C, 9. Cisplatin, 10. Etoposide, 11. ICRF-193, 12. Camptothecin, 13. Hydroxyurea, 14. Aphidicolin, 15. Thioguanine, 16. Mimosine, 17. Colcemid, 18. Trichostatin A.
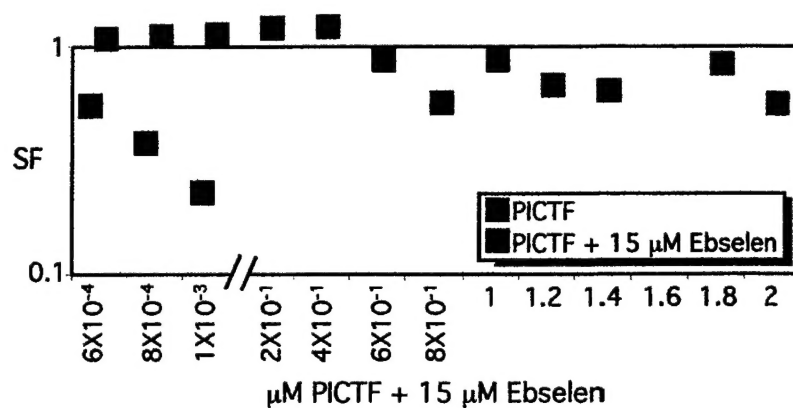
Figure 3. Dose response of ES cells to antp-PICTF and ebselen. The concentration of ebselen is constant at 15 μM (top). The concentrations of antp-PICTF are constant at 2 μM (bottom).
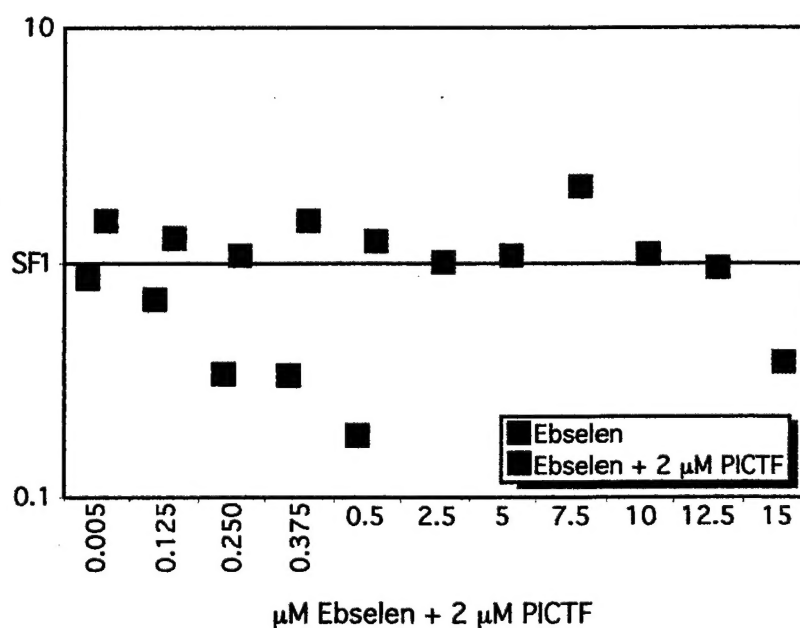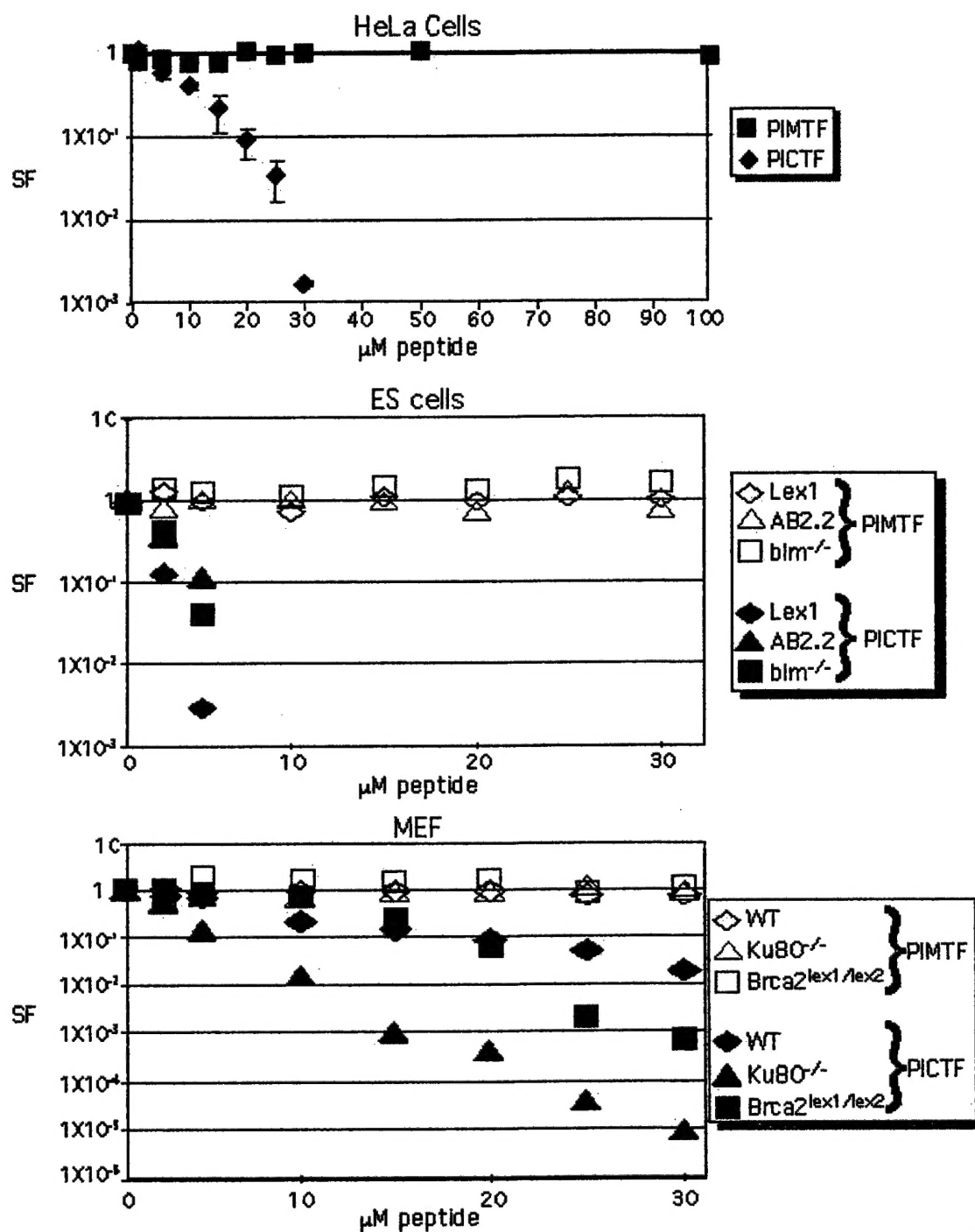
Figure 4. Dose response of antp-PICTF and antp-PIMTF to a variety of cells. HeLa cells are sensitive to antp-PICTF but not antp-PIMTF (top). ES cells are sensitive to antp-PICTF but not antp-PIMTF (middle). Two types of wild type (WT) ES cells were observed (lex1, Ab2.2). Both were derived from 129SvEv mice; Lex1 are Hprt positive while AB2.2 are Hprt negative. Blm-deficient cells were generated from AB2.2 ES cells. Mouse embryonic fibroblasts (MEF) are sensitive to antp-PICTF but not to antp-PIMTF (bottom). MEF deleted for NHEJ *(ku80^{-/-})* or deficient for homologous recombination $(brca2^{lex1/lex2})$ are hypersensitive to antp-PICTF.

Key Research Accomplishments

1) Established the genotoxin screen for ES cells, HeLa cells and MEF.
2) Ebselen and antp-PICTF are synergistic.
3) Cells deficient for repairing DNA double-strand breaks by mutating either Ku80 or Brca2 are hypersensitive to antp-PICTF.
4) Cells deficient for a RecQ helicase, Blm, are not hypersensitive to antp-PICTF.
5) Antp-PIMTF has no activity for any cell type tested, with or without ebselen.

Reportable Outcomes

Patent No.: 6,037,125
Title: Disruption of the Mammalian Rad51 Protein & Disruption of Proteins that Associate with Mammalian Rad51.

Patent No.: 6,057,104
Title: Disruption of the Mammalian Rad51 Protein & Disruption of Proteins that Associate with Mammalian Rad51 for Hindering Cell Proliferation

Conclusions

We are investigating the potential of a peptide, antp-PICTF, as an anticancer therapeutic. PICTF is five amino acids from exon 27 of Brca2 that interacts with Rad51. This peptide disrupts Rad51 foci and induces cell death. Changing the C to an M abolishes these activities; thus, the cysteine is critical. In addition, Ebselen greatly increases this toxicity again emphasizing the importance of the cysteine because ebselen reacts with thiols and in the presence of thiols, ebselen mimics the catalytic activities of phospholipid hydroperoxide glutathione peroxidase (10). Thus, it is likely that ebselen reduces the thiol group on cysteine. We have discovered that cells deficient for repairing DNA double-strand breaks are hypersensitive to antp-PICTF indicating that the peptide either induces breaks or impairs the repair of these breaks. The latter explanation is more likely since PICTF associates with Rad51. We are currently investigating the conformation of the peptide to determine if racemization is a factor in its activity. We will start to analyze the effect of the peptide on mice once we determine the reason why some batches of peptide have activity and others do not. We are also determining the dose response to a variety of cells, both cancer cells and more genetically altered cells.

References

1. **Chen, P. L., C. F. Chen, Y. Chen, J. Xiao, Z. D. Sharp, and W. H. Lee.** 1998. The BRC repeats in BRCA2 are critical for RAD51 binding and resistance to methyl methanesulfonate treatment. Proc Natl Acad Sci U S A **95:**5287-92.

2. **Connor, F., D. Bertwistle, P. J. Mee, G. M. Ross, S. Swift, E. Grigorieva, V. L. Tybulewicz, and A. Ashworth.** 1997. Tumorigenesis and a DNA repair defect in mice with a truncating Brca2 mutation. Nat Genet **17:**423-30.

3. **Donoho, G., M. A. Brenneman, T. X. Cui, D. Donoviel, H. Vogel, E. H. Goodwin, D. J. Chen, and P. Hasty.** 2003. Deletion of Brca2 exon 27 causes hypersensitivity to DNA crosslinks, chromosomal instability, and reduced life span in mice. Genes Chromosomes Cancer **36:**317-31.

4. **Lim, D. S., and P. Hasty.** 1996. A mutation in mouse rad51 results in an early embryonic lethal that is suppressed by a mutation in p53. Mol Cell Biol **16:**7133-43.

5. **Lim, D. S., H. Vogel, D. M. Willerford, A. T. Sands, K. A. Platt, and P. Hasty.** 2000. Analysis of ku80-mutant mice and cells with deficient levels of p53. Mol Cell Biol **20:**3772-80.

6. **Luo, G., I. M. Santoro, L. D. McDaniel, I. Nishijima, M. Mills, H. Youssoufian, H. Vogel, R. A. Schultz, and A. Bradley.** 2000. Cancer predisposition caused by elevated mitotic recombination in Bloom mice. Nat Genet **26:**424-9.

7. **Morimatsu, M., G. Donoho, and P. Hasty.** 1998. Cells deleted for Brca2 COOH terminus exhibit hypersensitivity to gamma- radiation and premature senescence. Cancer Res **58:**3441-7.

8. **Nakamura, Y., Q. Feng, T. Kumagai, K. Torikai, H. Ohigashi, T. Osawa, N. Noguchi, E. Niki, and K. Uchida.** 2002. Ebselen, a glutathione peroxidase mimetic seleno-organic compound, as a multifunctional antioxidant. Implication for inflammation-associated carcinogenesis. J Biol Chem **277:**2687-94.

9. **Patel, K. J., V. P. Yu, H. Lee, A. Corcoran, F. C. Thistlethwaite, M. J. Evans, W. H. Colledge, L. S. Friedman, B. A. Ponder, and A. R. Venkitaraman.** 1998. Involvement of Brca2 in DNA repair. Mol Cell **1:**347-57.

10. **Schewe, T.** 1995. Molecular actions of ebselen--an antiinflammatory antioxidant. Gen Pharmacol **26:**1153-69.

11. **Sharan, S. K., M. Morimatsu, U. Albrecht, D. S. Lim, E. Regel, C. Dinh, A. Sands, G. Eichele, P. Hasty, and A. Bradley.** 1997. Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2 [see comments]. Nature **386:**804-10.

12. **Sung, P., K. M. Trujillo, and S. Van Komen.** 2000. Recombination factors of Saccharomyces cerevisiae. Mutat Res **451:**257-75.

13. **Wooster, R., G. Bignell, J. Lancaster, S. Swift, S. Seal, J. Mangion, N. Collins, S. Gregory, C. Gumbs, and G. Micklem.** 1995. Identification of the breast cancer susceptibility gene BRCA2 [see comments] [published erratum appears in Nature 1996 Feb 22;379(6567):749]. Nature **378:**789-92.

14. **Zhu, C., M. A. Bogue, D. S. Lim, P. Hasty, and D. B. Roth.** 1996. Ku86-deficient mice exhibit severe combined immunodeficiency and defective processing of V(D)J recombination intermediates. Cell **86:**379-89.

Appendices